Scientific Research and Essays Vol. 6(28), pp. 5986-5992, 23 November, 2011
Available online at http://www.academicjournals.org/SRE
DOI: 10.5897/SRE11.877
ISSN 1992-2248 ©2011 Academic Journals

Full Length Research Paper

Microsatellite gene diversity within Philippines dwarf coconut palm (*Cocos nucifera* L.) resources at Port-Bouët, Côte d'Ivoire

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Accepted 18 October, 2011

Description and assessment of genetic resources are common preoccupations for their better management and utilization in breeding program. Progress made to date is still limited in view of assessment of coconut resources in the International Coconut Genebank for Africa and Indian Ocean (ICG-AIO) at Marc Delorme Port-bouët experimental station in Côte d'Ivoire. Using twelve microsatellite markers, we analyzed genetic diversity and genetic relationship among 25 palms representing five Philippines coconut green dwarf accessions. The 12 primers yielded 40 alleles, with an average of 3.33 alleles per primer. The within-accession genetic diversity (He) ranged from 0.067 ± 0.159 to 0.325 ± 0.205. In spite of the low level of polymorphism observed at microsatellite loci, factorial analysis of correspondence and hierarchical cluster analysis showed a clear distinction of aromatic green dwarf accession from others Philippines green dwarf accessions, which were not clearly separated. Indeed, there is no apparent partition between Kinabalan green dwarf and Catigan green dwarf individuals and between Pilipog green dwarf and Tacunan green dwarf accessions. Nevertheless, genetic differentiation indices ranged from 0.165 to 0.47 were obtained between these last accessions. The opportunity to consider these accessions as distinct each other relatively to their low genetic differentiation indices was discussed.

Keys words: Assessment, dwarf coconut accessions, microsatellites, Côte d'Ivoire.

INTRODUCTION

Côte d'Ivoire, through National Center for Agronomic Research (CNRA) coconut research program at Marc Delorme Port-Bouët, shelters the International Coconut Genebank for Africa and Indian Ocean (ICG-AIO). About ninety nine accessions compose this genebank. They were introduced from the whole coconut cultivation area.

This collection is the most important coconut genebank in the world. But, the genetic diversity of coconuts resources at Marc Delorme station is, to date, incomplete described. Indeed, description and assessment of the coconut resources in the ICG-AIO are currently undertaken for their better knowledge, management and utilization in the breeding program. Traditional approach used is based on morphological and agronomic traits (Le Saint et al., 1983; Sangaré et al., 1984; N'cho et al., 1988). Such a method is thwarted by environment plasticity which influences the traits under consideration. More recently, various molecular marker techniques such as RFLP (Lebrun et al.1998a, b), RAPD (Ashburner et al., 1997; Upadhyay et al., 2004), AFLP (Perera et al., 1998; Teulat et al., 2000) and SSR (Perera et al., 2003;

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Table 1. Name of accessions study, their code and country of origin.

Names of dwarf accessions	Accession code*	Country of origin	Number of palm
Catigan green dwarf	CATD'80	Philippines	5
Tacunan green dwarf	TACD'82	Philippines	6
Philipog green dwarf	PILD'82	Philippines	4
Kinabalan green dwarf	KIND'82	Philippines	5
Aromatic green dwarf	AROD'80	Philippines	5

*Symbol used are internationally recommended and numbers which were associated with them translate their field establishment.

Table 2. Number of alleles and level of gene diversity detected for 12 SSRs primer pairs.

Locus name	Number of samples	Number of alleles	Gene diversity index (He) ± SD
CnCirA3	25	2	0.084 ± 0.084
CnCirA9	25	4	0.232 ± 0.096
CnCirB6	25	3	0.187 ± 0.118
CnCirC7	25	3	0.192 ± 0.085
CnCirC12	25	5	0.377 ± 0.124
CnCirE2	25	3	0.132 ± 0.081
CnCirE10	25	2	0.192 ± 0.085
CnCirE12	25	2	0.156 ± 0,102
CnCirF2	25	5	0.200 ± 0.122
CnCirG11	25	2	0.255 ± 0.105
CnCirH4	25	6	0.335 ± 0.096
CnCirH7	25	3	0.268 ± 0.111
Mean ± SD	25	3.33 ± 0.396	0.218 ± 0.024

Meerow et al., 2003) were developed for coconut genetic diversity analysis. But, only a few methods, such microsatellite gene diversity analysis technology, offer powerful alternative in networked efforts across several continents consistent with a Coconut Genetic Resources Network (COGENT) strategy.

Coconut microsatellite gene diversity analysis technology, financially supported by the International Plant Genetic Resources Institute (IPGRI, now Bioversity International) and the Coconut Genetic Resources Network (COGENT), was transferred to Côte d'Ivoire (konan et al., 2007) for ICG-AIO resources characterization, since 2002.

In this paper we used microsatellite markers to assess genetic diversity within Philippines coconut Green Dwarf accessions conserved in ICG-AIO at Marc Delorme Port-Bouët coconut research station in Côte d'Ivoire.

MATERIALS AND METHODS

Plant materials and experimental site

The present investigation was conducted in 2007 at the Central Biotechnology Laboratory of CNRA (National Center for Agronomic Research). The material analysed was composed of 25 trees from five Philippines coconut green dwarf accessions. These accessions are all maintained at the International Coconut Genebank for Africa and Indian Ocean at Marc Delorme Port-Bouët in Côte d'Ivoire (Table 1).

DNA extraction

Total genomic DNA was extracted from fresh coconut leaflet following procedure derived to a MATAB protocol described by Risterucci et al. (2000). In our experimentation, tube was centrifuged at 4000 g during fifteen (15) min and DNA was re-suspended in 1 ml sterilized distilled water instead of 7000 g during thirty (30) min and 1 ml TE buffer in Risterucci et al. (2000).

Microsatellite analysis

PCR amplification was performed in 10 µl reaction mixture containing 25 ng template DNA, 10 mM Tris, 50 mM KCl, 2.25 mM MgCl2, 0.001% glycerol, 200 µM of each dNTPs, 0.2 µM of each primer (Table 2) and 1 unit taq polymerase. Reactions were covered with one drop of mineral oil. PTC 100 thermal cycler was used for amplification. The PCR regime consisted of an initial denaturation (94°C) for 5 min, 35 cycles each consisting of 30 s denaturation (94°C), 1 min annealing (51°C), and 1 min elongation (72°C). At the end of the final run, an extension period of 30 min at 72°C was observed. The primers used were developed by CIRAD

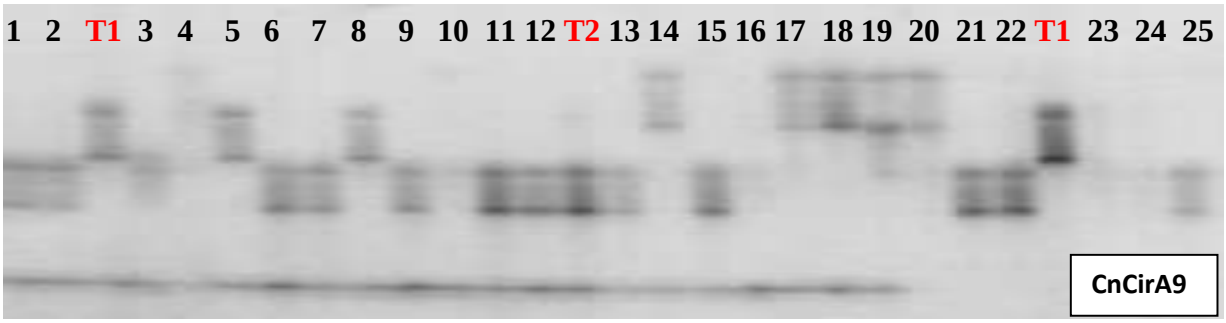

Figure 1. An example of allelic polymorphism at microsatellite locus CnCirA9 in 25 individuals of five Philippines coconut green dwarf accessions. Number 1-5 (CATD'80), 6-11 (TACD'82), 12-15 (PILD'82), 16-20 (KIND'82), 21-25 (AROD'80), T1 and T2 are standard samples used for alleles size determination.

(International Cooperation Center for Research and Development) at Montpellier in France and described by Baudouin and Lebrun (2002). The PCR products were separated on 5% acrylamide gels. The gels were run at constant power of 55 w for 2 h in 1X TBE buffer. The products were then revealed using a silver staining method as described by Creste et al. (2001).

Statistical analyses

Two kinds of matrices were generated for statistical analyses. In the first matrix, SSRs banding patterns revealed were coded using allele sizes. Each band size was estimated according to its comparative position to two references bands of coconut DNA (T1 and T2) used as control in each SSR essay**.** This matrix was used for SSR genetic polymorphism, within-accessions genetic diversity and factorial analysis of correspondence (FAC) computed. These data were processed by GENETIX v.4.05.2. Genetic polymorphism at each SSR locus was quantified using the number of alleles detected at each locus and the gene diversity, according to Masatoshi (1973) as reported by Tang et al. (2007). The mean number of allele per locus (A), observed heterozygosity (Ho) and gene diversity (He) were used to measure within genetic diversity parameters for each Philippines coconut green dwarf accession. Genetic structure and relationship among the 25 individuals tested was also visualized through a hierarchical cluster analysis method based on the unweighted pair-group method with arithmetic averages (UPGMA) computed on STATISTICA v6 program. Cluster analysis was applied on Jaccard's dissimilarity indices matrix computed from the second matrix of data based on scoring microsatellite bands as present (1) or absent (0) among individuals. Jaccard's dissimilarity indices were calculated using XLSTAT v. 7.5. program. Molecular differentiation index through an analysis of molecular variance (AMOVA) and accession pairwise differentiation (Fst) were moreover calculated. These parameters estimated the genetic variability partition and the genetic differentiation degree between accessions studied. The number of permutations for significance testing of pairwise differentiation (Fst) was set at 100. These last analyses were done using the ARLEQUIN Vs 3.1 program.

RESULTS

SSRs polymorphism

Microsatellite loci were all polymorphic through the whole sample tested. But, all twelve microsatellite loci used were weakly polymorphic (Figure 1). The twelve microsatellite primer pairs produced a total of 40 alleles ranging from 2 alleles for microsatellite primer pairs CncirA3, CncirE10, CncirE12 and CncirG11 to 6 alleles for microsatellite primer pair CncirH4 (Table 2). The mean number of alleles per locus was 3.33 ± 0.396. This translated feeble gene diversity at the microsatellite loci, with an average gene diversity index of 0.218 ± 0.024 (Table 2). The lowest molecular variability was obtained at the locus CncirA3, whereas the highest one was it at the microsatellite locus CncirC12, with gene diversity index of 0.084 ± 0.084 and 0.377 ± 0.124 respectively.

Intra accession gene diversity

The weakest allelic diversity was evidenced with aromatic green dwarf accession for which the mean number of alleles was 1.17. The strongest one was it with Catigan green dwarf for which the mean number of alleles was 2.00 per locus. On average, the allelic diversity amongst the Philippines Green Gwarf accessions (1.68 ± 0.14) was very low (Table 3).

The gene diversities (He) were relatively low for all accessions (Table 3) and ranged from 0.067 ± 0.159 for aromatic green dwarf accession to 0.325 ± 0.205 for Catigan green dwarf. The pooled gene diversity (He) was 0.218 ± 0.044. In general the observed heterozygosity (Ho) levels were proportional to the gene diversity values (Table 3). The observed heterozygosity ranged from 0.033 for aromatic green dwarf accession to 0.233 for Catigan green dwarf. Overall mean number of the observed heterozygosity was 0.098 ± 0.035. Otherwise, the mean number of the observed heterozygosity (0.098 ± 0.035) was lower than gene diversities (0.218 ± 0.044) which also translated the mean number of expected heterozygosity. This deficit of observed heterozygosity compared to the expected heterozygosity translated deviations from the Hardy Weinberg expectation within the Dwarf accessions tested.

Table 3. Genetic variation within five Dwarf coconut accessions from Philippines.

Accessions	Number of samples	Mean number of alleles per locus (A)	Observed heterozygosity (Ho)	Gene diversity index (He) ± SD
Catigan green dwarf	5	2.00	0.233	0.325 ± 0.205
Tacuna green dwarf	6	1.83	0.097	0.256 ± 0.251
Philipog green dwarf	4	1.58	0.062	0.180 ± 0.170
Kinabalan green dwarf	5	1.83	0.067	0.260 ± 0.240
Aromatic green dwarf	5	1.17	0.033	0.067 ± 0.159
Mean ± SD		1.68 ± 0.14	0.098 ± 0.035	0.218 ± 0.044

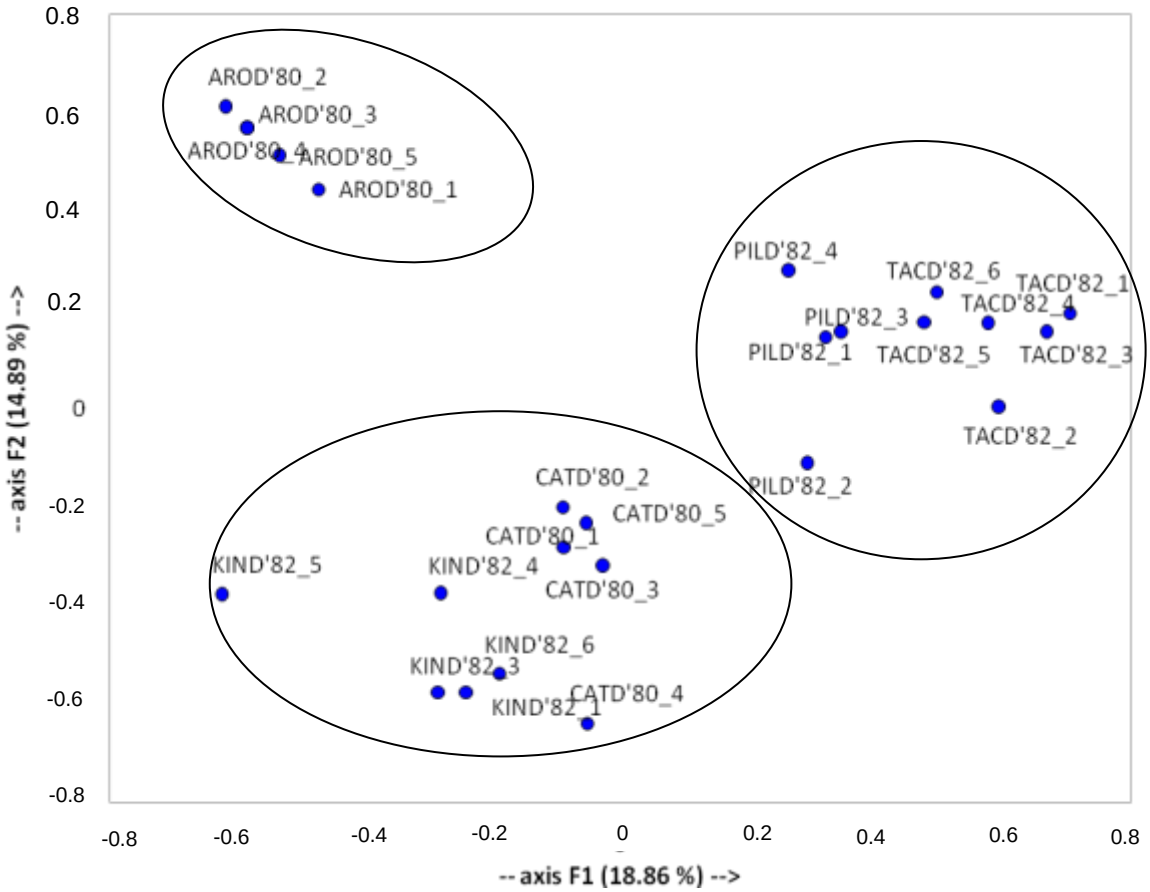

Figure 2. Structure of 25 screened coconut palms belonging to five coconut green dwarf accessions on the 1-2 plan of FAC performed on twelve microsatellite markers.

Genetic difference between accessions

The FAC was performed on the 25 individuals constituting the five Philippines green dwarf accessions using twelve microsatellite loci (Figure 2). The first two components from FAC contributed to 18.86% and 14.89% variations, respectively. The two principal components revealed to a certain extent the within- and between-accession variations. Mainly, the Philippines aromatic green dwarf accession was clearly stood out from others Philippines green dwarf accessions in the first two components. These ones were accounted for 33.75% of the total variation (Figure 2). In the same way, Kinabalan green dwarf and Catigan green dwarf individuals were grouped as Pilipog green dwarf and Tacunan green dwarf samples. These two sub-groups were feeble different each other (Figure 2). A dendrogram based on Jaccard's dissimilarity indices

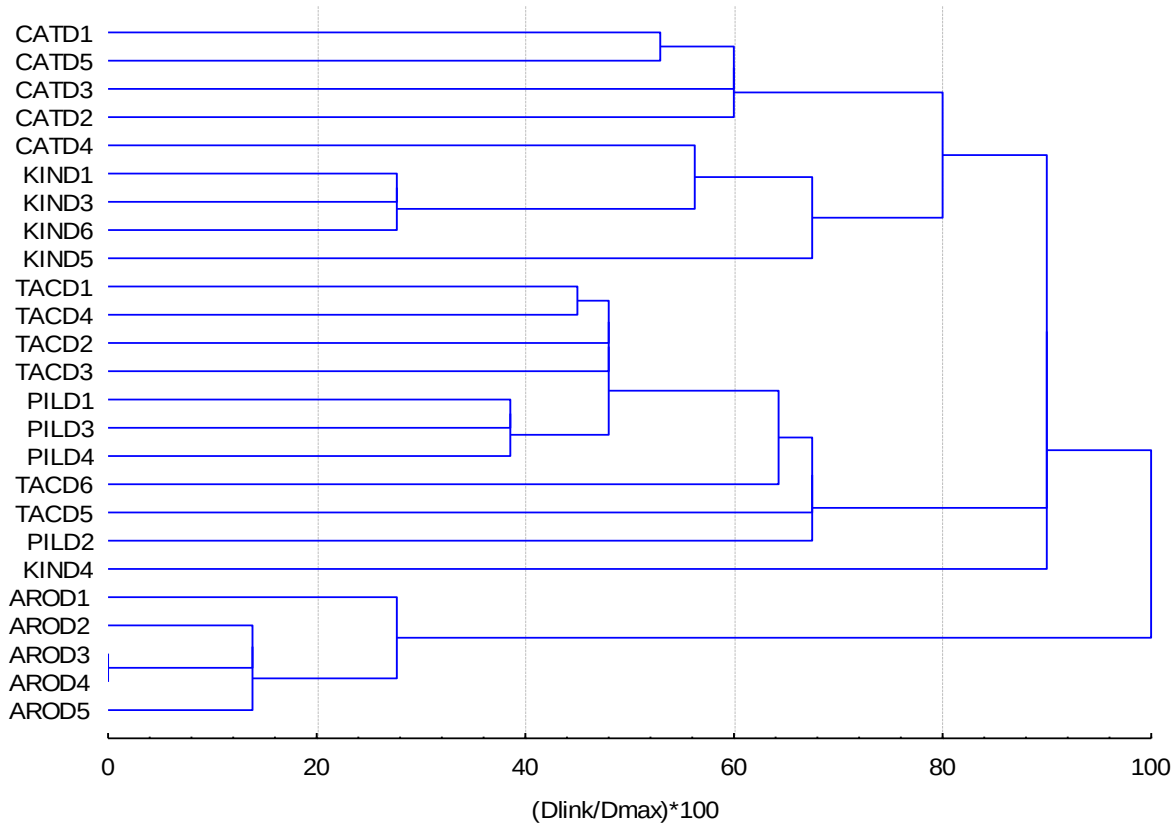

Figure 3. Genetic relationship between Philippines coconut green dwarf individuals resulting from hierarchical cluster analysis of microsatellites based on Jaccard's dissimilarity Indices matrix.

Table 4. molecular differentiation index of five Philippines coconut green dwarf accessions through the AMOVA analysis based on twelve microsatellite markers.

Source of variation	d.f	Sum of squares	Variance components	% variation	P value
Among groups	2	53.610	1.15674	33.90	0.0645
Among accessions within groups	2	16.250	0.53776	15.76	< 0.0001
Among individuals within populations	20	57.100	1.13370	33.34	< 0.0001
Within individuals	25	14.500	0.58000	17.00	< 0.0001

between accessions confirmed the FAC results (Figure 3).

AMOVA analysis indicated that 49.66% and 50.34% of the genetic variation were partitioned among accessions and within individuals, respectively (Table 4). This analysis also showed that the partition of accessions in three sub-groups, supported by 33.90% of allele variation, with 1023 permutations test was not significant. But, proportions of allele variation among accessions within groups (15.60 %), among individuals within accessions (33.34%) and within global population (17.00%) are supported by a high significant test (Table 4).

Fst Pairwise genetic difference indices were estimated to evaluate differentiation between the accessions. The pairwise genetic difference indices, computed among accessions, ranged from 0.165 to 0.714. Genetic differentiation between Philippines Aromatic Green Dwarf accession and the others was translated by more than 60% of allele variability between accessions (Table 5). All others Philippines Green Dwarf accessions were more similar, with pairwise genetic divergence lower than 0.50

Table 5. Pairwise genetic difference (Fst above diagonal) between Philippines coconut green dwarf accessions and Fst P values (below diagonal) obtained after 100 permutations of individuals between accessions to test significance of the divergences observed (α = 0.05).

	CATD'80	TACD'82	PILD'82	KIND'82	AROD'80
CATD'80	-	0.374	0.401	0.312	0.617
TACD'82	0.0090	-	0.165	0.464	0.653
PILD'82	0.0090	0.0450	-	0.470	0.714
KIND'82	0.0000	0.0000	0.0180	-	0.665
AROD'80	0.0180	0.0000	0.0270	0.0090	-
	Mean Fst ± sd			0.463 ± 0.058	

(Table 5). Pilipog Green Dwarf and Tacuna Green Dwarf accessions were closest, with a pairwise genetic difference index of 0.165. The re-sampling test, with 100 permutations of individuals between accessions, indicated that all pairwise genetic difference indices were significant (α = 0.05) solid (Table 5).

DISCUSSION

Genetic diversity within Philippines dwarf coconut accessions was analyzed. In previous coconut populations genetic diversity studies, using isoenzyme markers (Zizumbo et al., 2000), RFLPs markers (Lebrun et al., 1998a), RAPDs markers (Upadhyay et al., 2004) and SSRs markers (Perera et al., 2003), low genetic diversity was reported on coconut dwarf accessions. The present study corroborated these results. Sure enough, a low level of polymorphism was also detected within Philippines dwarf accessions tested through twelve microsatellite markers (A = 1.68 ± 0.14; He = 0.218 ± 0.044; Ho = 0.098 ± 0.035). This relatively low within accession diversity could be a consequence of the dwarf populations fertilization system. Indeed, the dwarf coconut cultivars are known to be mainly self-pollinated (Sangaré et al., 1978). So, these populations were predisposed to fix randomly alleles at each locus and consequently have homogeneous genetic constitution.

In spite of a low within genetic diversity observed, factorial analysis of correspondence and hierarchical cluster analysis performed on the whole microsatellite markers showed partition of Philippines green dwarf accessions into three sub-groups. Aromatic green dwarf accession is clearly distinct from others accessions. Pilipog green dwarf and Tacuna green dwarf accessions formed one group whereas Kinabalan green dwarf and Catigan green dwarf accessions were clustered together. Similar results were previously reported by Meerow et al., *(2003*). These authors successfully used microsatellite markers to demonstrate that the Malayan Red Dwarf is genetically distinct from the Malayan Green and Yellow Dwarf accessions, which cannot be distinguished. But, Zizumbo et al. (2000) and Upadhyay et al. (2004), using enzymatic and RAPD markers, respectively, reported that Dwarf coconut accessions from geographically distant regions did not cluster separately. So, genetic characterization of coconut accession using microsatellite markers allowed the detection of detailed genetic differentiation between accessions.

The partitioning of variation within and between accessions computed by AMOVA procedure, showed an approximate equality of distributed variation between (49.66%) and within (50.34%) accessions of Philippines coconut Green Dwarfs. Perera et al. (1998) cited by Perera et al. (2001) obtained also a globally equally distributed variation between and within forms of tall coconuts using the same statistic analysis procedure. The Fst indices calculated to estimate differentiation between accessions varied from 0.165 (16.5%) to 0.714 (71.4%), with a mean number of 0.463 (46.30%). These results reflected a moderate level of population differentiation. Nevertheless, the value was statistically significantly for population differentiation. Risterucci *et al*. (2000) suggested that when the DNA profile is different for at least one locus, the genotypes tested are considered different. The Fst value indicates segregation between genotypes due to the fixation of dissimilar allele by the genotypes.Values for Fst ranged from 0, for non-differentiation, to 1 for complete differentiation between an original group and its sub-groups (Herna'n, 2009). In the same way, we can consider that the genotypes tested are different if Fst index estimated is superior to zero. Therefore, the accessions analyzed can be considered as distinct, even if feeble molecular differentiations were observed between some ones.

In general, our study demonstrated lower genetic diversity within Philippines Dwarf accessions. However, differences were observed between some accessions. Indeed, the Philippines Aromatic Green Dwarf accession showed an important genetic difference from others Philippines Green Dwarf accessions. This information is important for ongoing efforts to preserve coconut resources and to develop high yielding hybrids. This study also confirmed the efficacy of SSR markers to revealed diversity and genetic relationship in the germplasm. Hence, analysis deserves to be deepened by

using markers covering the entire genome of the coconut to refine the results.

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